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Tissue Engineering of Fibroblast Constructs and Anisotropic Collagen Gels

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ABSTRACT

The creation of an *in vitro* functional tendon construct will enable testing of the influence of mechanics and nutrients on the development and remodeling of tendon under known controlled stimuli which is difficult to achieve *in vivo*. Tendon constructs were engineered *in vitro* via stress-mediated self organization of fibroblasts and ECM on a laminin coated elastomer substrate. Varying the laminin density and the amount of fetal bovine serum on the substrate affected the ability of tendon fibroblasts to form a confluent cell layer and the time to layer delamination. Understanding the factors that promote self-assembly of tendon constructs will enable their combination with already developed *in vitro* muscle constructs.

INTRODUCTION

Self-assembled muscle constructs, termed myooids, have been formed without a synthetic scaffold via stress-mediated self-organization [1]. Myooids can transmit force spontaneously and upon the application of an electrical stimulus. They display an immature phenotype both morphologically and mechanically however, which hinders their use as a model of *in vivo* muscle function. Mechanical stress is one of the mechanisms that has been shown to facilitate muscle maturation [2], but the interface between the anchor, medical silk suture, and the myooid is not strong enough to withstand stress on the physiological scale. This problem may be ameliorated by creating, *in vitro*, the natural interface muscle uses to transmit generated force. (Figure 1) The myotendinous junction is highly infolded increasing the interfacial area between the

Figure 1. TEM of a neonatal myotendinous junction from a 2 day old Fischer 344 rat (x10,140). The muscle cell is coming out of the lower right hand corner and is outlined by dark, electron rich areas which demarcate areas of adhesion between the fibroblasts and collagen of the tendon and the muscle cell. Note the involuting membrane. These will become more pronounced during development as the unit is put under more stress. The wavy lines in the central portion of the picture represent collagen fibrils of the tendon. Photo: Krystyna Pasyk

tendon and muscle, allowing for the tension to be transmitted primarily via shear. A critical step towards myotendinous junction formation *in vitro* is the development of an independent tendon construct. After understanding the factors that facilitate tendon formation, myoids and tendon constructs can be combined by co-culturing tenoblasts and myoblasts or by growing the constructs independently of each other and inducing the two constructs to combine.

EXPERIMENTAL DETAILS

Acquisition of tendon fibroblasts

Primary rat tendon fibroblasts were obtained by surgically removing the Achilles tendon from a Fischer 344 rat and dissociated by placing in a 0.25% trypsin-EDTA solution (GIBCO BRL, Rockville, MD 25200-027) containing 100 units/mL type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ 4196) at 37°C for several hours. After tissue was dissociated, the solution was centrifuged and the supernatant was suctioned off. The cells were reconstituted with growth medium (GM): 400 mL Ham F-12 (GIBCO BRL 11765-054), 100 mL fetal bovine serum (FBS) (GIBCO BRL 10437-036), 100 units/mL antibiotic-antimycotic (Sigma, St. Louis, MO A9909) and expanded in 100 mm diameter tissue culture dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ 353003). Cells were passaged at 60% confluence and kept in LN_2 until needed for experimentation.

Preparation of culture dishes

1.5 mL SYLGARD (Dow Chemical Corporation, Midland, MI type 184 silicone elastomer) was poured into each 35 mm diameter culture dish (Falcon 351008). After curing for at least 2 weeks, the dishes were rinsed with Dulbecco's Phosphate-Buffered Saline (DPBS) (pH = 7.2 GIBCO BRL 14190-136) and coated with varying densities of natural mouse laminin (GIBCO BRL 23017-015) by diluting the laminin in ~3 mL DPBS and allowing the DPBS to evaporate overnight. The dishes were rinsed with DPBS and the anchors, 6 mm segments of size-0 silk suture (Ethicon, metric size 3.5), were dipped into 50 μg/mL solution of natural mouse laminin, then pinned 12 mm apart with 0.10 mm diameter stainless steel minutien pins (Fine Science Tools, Foster City, CA, 26002-10). The sutures were allowed to dry and the plates were filled with ~2 mL GM, enough to cover the top of the sutures, and the plates were sterilized with ultraviolet irradiation (253.7 nm, bulb G30T8) in a biological safety hood for 90 minutes. The sterilized plates were placed in an incubator (5% CO_2, 37°C) for 5-8 days.

When the plates were ready, $1x10^5$ cells were added to each plate and grown in 2mL GM. After two days, 100 μg/mL of ascorbic acid (GIBCO BRL 13080-023) was added to each dish. Fresh ascorbic acid was added to the dishes when the media was changed, every 2-3 days. After 2 or 5 days, the media was changed from GM to differentiation media (DM): 465 mL DMEM (GIBCO BRL 11995-065), 35 mL FBS, 100 units/mL A9909.

Preparation of anisotropic collagen constructs

6 mL of 2 mg/mL collagen solution was made by combining 0.6 mL 10x MEM (GIBCO BRL 11430-030), 0.06 mL NaOH (1.0 N), 2.56 mL DI water and 2.78 mL type I rat tail collagen solution (BD Biosciences, Bedford, MA 354236 4.31 mg/mL) in that order. The pH was adjusted to 7.4 by the addition of 0.1 N acetic acid or NaOH. A cell solution was made by suspending tendon fibroblasts in GM at a concentration of $1x10^5$ cells/mL. Gels were formed by mixing 1.0 mL cell solution with 1.0 mL collagen solution and poured into a UV treated 35 mm diameter plate with anchors but without laminin coating.

DISCUSSION

Self assembling tendon constructs

Cells that have reached confluence before plating on prepared substrates do not form confluent layers. (Figure 2b) This is likely a result of contact inhibition in which the cells become quiescent, losing their ability to proliferate when dissociated and re-plated. Cells should not be allowed to become more than 60% confluent when expanding.

Ascorbic acid is utilized during the synthesis of collagen [3], and it is necessary for the formation of confluent cell layers on the laminin coated substrates used in these studies. The extra collagen synthesized in cultures supplemented with ascorbic acid probably acts as scaffold, maintaining the integrity of the cell layer. Without the addition of ascorbic acid, the cells dissociate from the substrate in a spatially sporadic manner. (Figure 2c)

When the fibroblasts are grown in 20% FBS, they will only delaminate when the substrate is coated with 2.0 mg/cm^2 laminin. At lower laminin concentrations, the fibroblasts will not form a confluent monolayer and at concentrations higher than 2.0 mg/cm^2, the cell layer will not delaminate. But, when the cells are initially grown in GM (20% FBS) then changed to DM (7% FBS), the cell layer delaminates for all laminin densities studied, 2.0-20.0 mg/cm^2.

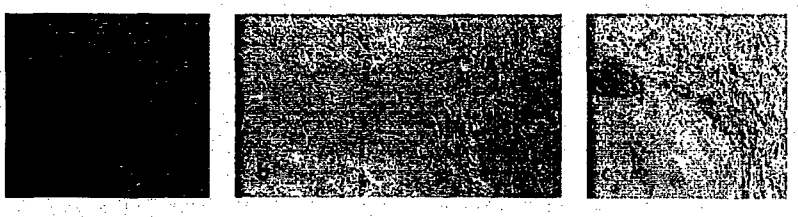

Figure 2. Rat tendon fibroblasts grown under various conditions. **A**: Confluent fibroblast layer which was plated with cells less than 60% confluent and supplemented with 100 µg/mL ascorbic acid. **B**: Cells that reached confluence before plated on laminin coated substrates could not form a confluent cell layer. **C**: Inability of cells to form confluent layer without addition of ascorbic acid. (100x magnification)

When the media was changed from GM to DM on day 3, the layer began to delaminate after 9 days. It formed a cylinder bounded by the anchors after 10-11 days, but the construct was too fragile and fractured. The extracellular matrix (ECM) secreted by the cells was not strong enough to maintain the integrity of the layer as the cells contracted or tried to adhere to one another. If the culture medium was changed to DM after 5 days instead, the cell layer started detaching after ~2.5 weeks and was much more stable. The higher percentage of FBS in GM, 20%, is hypothesized to facilitate the production of ECM significantly more than the 7% FBS in DM due to an increase in nutrients, creating a stronger cell layer.

Fibroblasts are believed to preferentially adhere to each other rather than the artificial substrate since there is only one adhesion ligand on the substrate and there are multiple adhesion proteins present on the cell membranes as well as the secreted ECM. When delamination starts, the fibroblast layer rolls up on itself and the fibroblasts preference for the cell layer acts as a catalyst for self assembly into a cylinder bounded by the anchors.

Cell-collagen constructs

The combination of cells and collagen as described in the experimental section is a simple and repeatable way to create fibroblast constructs. This construct more closely mimics the physiology of adult tendons than pure tendon cell constructs since there is a larger initial ratio of collagen to cells, and the fibroblast density can be easily controlled by varying the seeding density. Alternate anchor materials are being investigated for use with these collagen constructs since they do not adhere well to the silk suture (as seen in the top suture in figure 4). Porous polyethylene (PE) is an inert polymer currently used in

Figure 3: A: 19 days after plating. The cell layer has started to detach from the substrate. **B**: 19 days after plating. This picture was taken 3 hours after the media was changed and there is considerably more detachment evident. Whether or not this is a result of a fresh supply of FBS, which has been implicated in promoting fibroblast contraction [4], or a response to the mechanical stimulus of the addition of fluid to the plate is still unknown and an investigation of this phenomenon is planned. **C**: 21 days after plating. The increase in ECM allows for the contracted cell layer to maintain the connection between the anchors.

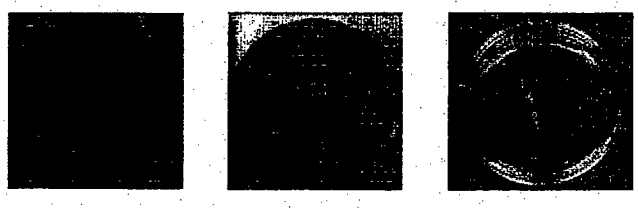

Figure 4. Anisotropic collagen-fibroblast constructs are easily formed over the course of a week. **A**: 12 hours after plating. **B**: 3 days after plating. **C**: 8 days after plating. The force of contraction is causing the gel to pull up from the anchor at the top of **C**.

medical applications. The inherent hydrophobic nature of PE can be overcome by introducing functional peroxide groups to the surface. These peroxides can then be reacted with chemicals that bind to collagen [5]. The use of plasma discharge treatment to create the desired interface that will bind with collagen is currently under investigation.

CONCLUSION

Self-assembled and collagen-based fibroblast constructs have been created. The knowledge gained from varying certain parameters (laminin density, % FBS, state of cell before plating, ascorbic acid concentration) will allow for control of the length of time it takes for delamination, as well as the collagen composition of the construct, to more closely mimic tendon physiology. One future direction is the combination of the self-assembled tendons with the collagen constructs in which the collagen construct will form a relatively acellular core that the cell layer wraps around.

It is anticipated that these constructs will be able to combine with previously developed myooids. It has been shown that when myoblasts are cultured adjacent to a type I collagen gel, the developing myotubes invaginate the collagen creating a structure similar to that seen in a myotendinous junction [6]. Myooids are expected to form this type of interface with the three-dimensional tendon constructs described above since tendon cells predominantly secrete type I collagen [7]. Further support comes from developmental studies showing that tendon and muscle initially grow independent of each other and only in later development combine in a non-site specific way [8].

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